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The microbiome of the footrot lesion in Merino sheep is characterized by a persistent bacterial dysbiosis

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ABSTRACT

Footrot is prevalent in most sheep-producing countries; the disease compromises sheep health and welfare and has a considerable economic impact. The disease is the result of interactions between the essential causative agent, *Dichelobacter nodosus*, and the bacterial community of the foot, with the pasture environment and host resistance influencing disease expression. The Merino, which is the main wool sheep breed in Australia, is particularly susceptible to footrot. We characterised the bacterial communities on the feet of healthy and footrot-affected Merino sheep across a 10-month period via sequencing and analysis of the V3-V4 regions of the bacterial 16S ribosomal RNA gene. Distinct bacterial communities were associated with the feet of healthy and footrot-affected sheep. Infection with *D. nodosus* appeared to trigger a shift in the composition of the bacterial community from predominantly Gram-positive, aerobic taxa to predominantly Gram-negative, anaerobic taxa. A total of 15 bacterial genera were preferentially abundant on the feet of footrot-affected sheep, several of which have previously been implicated in footrot and other mixed bacterial diseases of the epidermis of ruminants. Some, including *Porphyromonas*, can trigger a shift in the composition of bacterial communities and may therefore be of significance to the expression of footrot. A comparison of the communities in footrot lesions of different scores and at different times revealed that this state of dysbiosis is persistent at the active margins of lesions, irrespective of their severity.

1. Introduction

Footrot is prevalent in most sheep-producing countries including Australia (Lane et al., 2015), where the disease is responsible for significant economic losses, and compromises sheep health and welfare (Lane et al., 2015). Footrot presents as a spectrum of clinical entities, from interdigital dermatitis through to separation of the horny epidermis from the underlying dermal tissues of the foot, resulting in lameness (Beveridge, 1941).

The clinical manifestations of footrot are the result of complex synergistic interactions between the essential causative agent, *Dichelobacter nodosus*, and the bacterial community of the foot (Beveridge, 1941; Stewart, 1989). The severity of these manifestations differs according to the virulence of the infecting *D. nodosus* strain(s) (Stewart et al., 1986), the susceptibility of the host (Emery et al., 1984), and environmental conditions, with mild air temperatures and high, evenly distributed rainfall favouring disease progression (Graham and Egerton, 1968).

The bacterial community of the ovine foot is diverse (Calvo-Bado et al., 2011; Maboni et al., 2017; Stewart, 1989); establishing which taxa contribute to the expression of footrot and the extent to which they contribute is challenging. The existing literature is primarily focussed on interactions between *D. nodosus*, *Fusobacterium necrophorum*, and *Trueperella pyogenes* (formerly *Corynebacterium pyogenes*) (Stewart, 1989).

Much of what is currently known about the bacterial aetiology of ovine footrot was ascertained using classical microbiological techniques and examination of histological sections (Beveridge, 1941; Egerton et al., 1969; Roberts and Egerton, 1969). Such studies are inherently limited to bacterial taxa that can be cultured on common growth media, or those with distinctive morphologies that are readily identifiable in smears or histological sections. However, the advent of new technologies such as next-generation sequencing (NGS) and amplicon-based metagenomics has enabled new insights into the aetiopathogenesis of complex polymicrobial diseases like ovine footrot, periodontal disease, psoriasis, and the persistence of chronic wounds (Borsanelli et al., 2018; Calvo-Bado et al., 2011; Maboni et al., 2017; Martin et al., 2010; Stehlikova et al., 2019).

A recent study undertaken in the U.K. found several genera, including *Treponema*, *Porphyromonas*, and *Anaerococcus* to be significantly

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more abundant on the feet of footrot-affected sheep (Maboni et al., 2017). The same study reported that an abundance of these three genera was associated with an increased expression of pro-inflammatory cytokines in the interdigital skin of sheep with underrun footrot lesions. The results of such studies have opened new avenues of investigation that may eventually yield new control and treatment measures. For example, the control or elimination of bacterial genera thought to trigger a severe inflammatory response, by means of a vaccine, could considerably reduce the severity of the disease.

The Merino, which is the main wool sheep breed in Australia, is particularly susceptible to footrot (Emery et al., 1984). The reason for this variation in innate resistance between breeds is uncertain, but several factors are thought to contribute, including variations in the magnitude and duration of immune responses to bacterial invasion (Emery et al., 1984), and variations in the structure of the epidermis (Emery et al., 1984). The interdigital skin of the Merino foot appears to be more susceptible to bacterial invasion than that of British breeds, such as the Border Leicester (Emery et al., 1984). Perhaps the foot of the Merino may be colonised by a greater number or diversity of opportunistic pathogens following environmental predisposition than British breeds, or the bacterial taxa of interest may differ in this breed. To our knowledge, the aetiology of footrot in this breed has not yet been investigated using amplicon-based metagenomics. Pioneering studies have previously been conducted in the United Kingdom but with samples collected from British sheep breeds only (Calvo-Bado et al., 2011; Maboni et al., 2017).

The aim of this study was to characterise the bacterial communities on the feet of healthy and footrot-affected Merino sheep using 16S ribosomal ribonucleic acid (rRNA) amplicon-based metagenomics, with the aim of determining which taxa are abundant in footrot lesions and might contribute to expression of the disease in this breed. Using a longitudinal study design involving repeated sampling of the same lesions across a 10-month period, we also examined the temporal stability of the bacterial community on footrot-affected feet.

2. Materials and methods

2.1. Trial

Merino sheep were sourced from flocks with footrot in New South Wales (NSW), Australia. The sheep ($n = 3$ sheep per mob) were selected by the authors from a proportion of the parent flock that had been drafted-off by the farmer because all had two or more feet with active footrot lesions. Group 1 was selected from a flock located near Crookwell, on the southern tablelands of NSW. Group 2 was selected from a flock located near Bombala, in south-eastern NSW. The sheep were transported separately to The University of Sydney at Camden, NSW one month prior to the commencement of the study. Healthy control Merino sheep ($n = 2$) were sourced from a flock with no history of footrot located near Camden. Each group of sheep was maintained in a separate $\sim 500\,m^2$ paddock, with a buffer zone approximately 10 m wide between paddocks. The pasture consisted of native and introduced species, and was dominated by kikuyu (*Pennisetum clandestinum*), paspalum (*Paspalum dilatatum*), pigeon grass (*Setaria geniculata*), and Rhodes grass (*Chloris guyana*). The pasture composition was similar throughout the paddocks. No ruminants had been grazed in the paddocks in the preceding two months, and no footrot-affected sheep had been in the paddocks in the preceding six-months. The sheep were monitored daily for signs of pain or distress (e.g. lameness and inappetence). In order to accurately characterise the bacterial communities on the feet of the sheep in the two footrot-affected sheep, no sheep in these groups were to be treated for footrot until the conclusion of the trial, unless they were showing signs pain or distress. All procedures were approved by the University of Sydney's Animal Ethics Committee (AEC Approval Number 2014/621). Weather data were drawn from the Australian Bureau of Meteorology (BOM) automatic weather station (AWS) located at Camden Airport (Station no. 68192), which is located approximately 2.5 km from the University of Sydney facilities.

2.2. Foot scoring

Each foot of each sheep was inspected before the trial, and then at approximately monthly intervals, and a score was assigned to each foot using a previously described scoring system (Egerton and Roberts, 1971). Briefly, mild lesions restricted to the interdigital skin were assigned a score of 1; if the interdigital lesion was severe, a score of 2 was assigned; where underrunning of the posterior sole and soft horn of the heel was observed, a score of 3 was assigned; if the underrunning extended to the abaxial hoof wall, a score of 4 was assigned. Feet were scored by the same operator at each time point, immediately prior to sample collection.

2.3. Study design and sample collection

Active foot lesions ($\geq$ score 1) that were suitable for sampling were identified in February 2016 when the sheep arrived at Camden. Samples were collected from the same feet each month for 10 months between March and December 2016. The study concluded in December and the sheep were treated. Samples were collected from the active margin of each lesion; i.e. the skin-horn junction for score 1 and 2 lesions or the margin of the underrun part of the lesion for score 3 and 4 lesions. Where there was no active lesion (score 0), samples were collected from the centre of the interdigital skin over a 1 cm wide and 3 cm long area. The sample was collected by firmly rubbing the head of the swab across the surface of the skin, and rotating the swab periodically to ensure that the entire head of the swab had contact with the epidermis. Two types of swab were used each time: a sterile, flocculated nylon swab (FLOQswabs; Copan Italia, Brescia, Italy) and a cotton-tipped swab (CLASSIQSwabs; Copan Italia, Brescia, Italy). The former (for DNA isolation), was placed in the individual plastic tube provided with the swab while the latter (for microbiological culture) was placed into modified Stuart's transport media (mSTM) (Amies, 1967).

2.4. Isolation of D. nodosus

D. nodosus was isolated from each lesion swab collected in mSTM, as described previously (Buller and Eamens, 2014).

2.5. Elastase test

The virulence of each *D. nodosus* isolate was assessed using the elastase test, as described previously (Stewart, 1979). Isolates that were elastase-positive at 12 days or less were deemed virulent. Isolates that were elastase-positive at 16–28 days, or not at all, were deemed benign.

2.6. DNA extraction

All nylon swabs were stored at $-80\,^{\circ}C$ prior to DNA preparation. DNA was prepared from each nylon swab using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany), with a mechanical prelysis step, following the protocol provided by the manufacturer for the preparation of microbial DNA from swabs. DNA was prepared from each pure culture of *D. nodosus* using the Wizard® Genomic DNA Purification Kit (Promega, Madison, U.S.A) in accordance with the protocol for the purification of genomic DNA from Gram-negative bacteria.

2.7. Evaluation of DNA quantity and quality

The quantity of double-stranded genomic DNA (dsDNA) prepared from each foot swab was analysed with a Qubit 2.0 fluorometer

(Invitrogen, Carlsbad, U.S.A.) using the Qubit dsDNA BR (Broad-Range) Assay Kit (Invitrogen, Carlsbad, U.S.A.) according to the manufacturer's instructions. The purity of each dsDNA extract was analysed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, North Ryde, Australia), following the manufacturer's instructions. dsDNA extracts that had a 260 nm/280 nm absorbance ratio ≥1.8 and a 260 nm/230 nm absorbance ratio ≥1.5 were deemed acceptable. dsDNA extracts that were below these thresholds were purified using a DNA clean-up and concentrator kit (DNA Clean-Up and Concentrator -5; Zymo Research, Irvine, U.S.A.), following the manufacturer's instructions.

2.8. Sequencing of the bacterial 16S rRNA gene

The V3-V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 319 F and 806R (Caporaso et al., 2011; Muyzer et al., 1993), and the products were sequenced on an Illumina MiSeq (Illumina, San Diego, U.S.A) using 300 base pair (bp) paired-end chemistry, by the Brisbane node of the Australian Genome Research Facility Ltd. (AGRF).

2.9. Analysis of bacterial community structure and diversity

The quality of the raw data set was assessed with FastQC v0.11.3 (Andrews, 2010). Paired-end reads were merged with USEARCH v10.0 (Edgar, 2010) using the default parameters: maximum of five mismatches in alignment; minimum 90% identity; and a minimum overlap of 16 bases. Quality filtering and diversity analyses were undertaken with Quantitative Insights into Microbial Ecology (QIIME) v2.7.10 (Caporaso et al., 2010b), available through The University of Sydney's High Performance Computing (HPC) service. Quality filtering was undertaken (Bokulich et al., 2013): minimum Phred scores of 3; a minimum of 75% consecutive high-quality bases per read; a maximum of three consecutive low-quality bases; and no ambiguous (N) characters. *De novo* and reference-based chimera detection was performed with USEARCH v6.1 (Edgar, 2010), as implemented in QIIME, against the GreenGenes 13_8 97% OTU database (McDonald et al., 2012). *De novo* operational OTUs were generated with a minimum sequence similarity of 97% using the 'UCLUST' algorithm (Edgar, 2010), as implemented in QIIME, and taxonomically classified against the GreenGenes 13_8 97% OTU database (McDonald et al., 2012). OTUs that represented < 0.005% of the total data set were discarded to avoid artificial inflation of diversity estimates due to the presence of spurious OTUs (Bokulich et al., 2013). A representative set of sequences was generated and aligned with PyNAST (Caporaso et al., 2010a). The alignment was filtered to remove gaps and variable positions with Lane masking and a phylogenetic tree was constructed with FastTree.

Prior to undertaking within-sample (alpha) diversity analyses, the data were rarefied with sampling depths between 10 and 40,000 reads per sample in steps of 2000 reads, with 10 iterations per step. Alpha diversity was assessed, as defined by the number of observed OTUs and Shannon's diversity index. Alpha diversities were compared between categories of interest (group, lesion score, time point) using a non-parametric *t*-test with 10,000 Monte Carlo permutations and Bonferroni corrected P-values. Between-sample (beta) diversity was assessed with a dataset rarefied to 20,000 reads using weighted and unweighted UniFrac distances (Lozupone et al., 2011). Principle coordinate analysis (PCoA) was performed on the resultant UniFrac matrices and the results were plotted with EMPeror, as implemented in QIIME. Changes in community structure between categories of interest (disease status, lesion score, time point) were compared using the non-parametric analysis of similarity (AnoSim) and ADONIS tests with 10,000 permutations.

Table 1
Foot scores for each foot sampled during this study. Scores were assigned to each foot using a previously devised scoring system (Egerton and Roberts, 1971). LH = left hind, RF = right fore, RH = right hind.

Group	Tag No. (Foot)	Foot score									
		Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Healthy	130 (LH)	0	0	0	0	0	0	0	*	*	*
	287 (RF)	0	0	0	0	0	0	0	*	*	*
1	317 (RH)	1	1	1	1	1	0	1	1	2	2
	318 (RF)	2	2	2	3	1	2	3	2	2	2
	318 (RH)	2	2	3	2	2	2	2	3	2	2
	319 (RH)	2	2	1	1	1	1	3	2	2	2
2	389 (RF)	2	2	3	2	1	1	2	2	4	3
	389 (RH)	2	2	3	2	1	2	2	2	2	2
	390 (RH)	1	0	0	1	1	1	2	1	3	3
	475 (RH)	2	2	0	1	1	1	2	2	2	2

2.10. Identification of biomarker taxa

Linear discriminant analysis effect size (LEfSe) was used to identify metagenomics biomarkers, which are defined as taxonomic groups that are preferentially abundant in a biological class of interest (Segata et al., 2011). Taxonomic count data generated in QIIME were analysed using LEfSE v1.0, available on the Galaxy server (http://huttenhower.sph.harvard.edu/galaxy/), using the default parameters.

3. Results

Lesion severity varied across the trial period for sheep with footrot, as shown in Table 1, probably associated with seasonal factors. The total monthly rainfall ranged from 13.2 mm to 63.2 mm during the trial period, with the exception of June 2016, in which 327 mm fell in the Camden area. Samples were collected from one foot of each healthy sheep (n = 2 sheep) and one or two feet of each footrot-affected sheep (n = 3 sheep per group, total 8 feet) each month (Table 1). The same feet were studied over time. *D. nodosus* was not isolated from the healthy group at any time point. *D. nodosus* isolates classified as virulent using the elastase test were present in Groups 1 and 2 together with isolates classified as benign. The study ran from March to December 2016. The control sheep were removed from the trial in September due to a louse infestation that required treatment. The trial was concluded in December as some sheep in the footrot-affected group were showing signs of lameness, and the sheep were treated by foot-bathing in 10% zinc-sulphate once per day for a period of four days. No sheep required treatment during the course of the trial.

16S rRNA sequences were obtained from all 80 swabs collected from the feet of sheep in Groups 1 and 2, and from all 14 swabs collected from the feet of sheep in the healthy group. The raw data set consisted of 8,179,973 paired-end reads with a length of 300 bp. After merging and quality filtering, the dataset consisted of 5,594,991 reads with an average length of 450 bp. The mean number of reads per sample was 59,521 (range 14,241 to 100,148). Sample metadata are reported in Supplementary Table 1.

D. nodosus was detected by amplification and sequencing of the V3-V4 region of the bacterial 16S rRNA gene and microbiological culture on the feet of all eight footrot-affected sheep but was not detected on the feet of the healthy sheep.

Within-sample (alpha) diversity was assessed after rarefaction, defined by the number of observed OTUs and Shannon's diversity index. Two samples from Group 1 (26, 50), and three samples from the Healthy group (83, 89, and 86) were excluded from all diversity analyses due to an insufficient number of reads (< 40,000). The rarefaction curves depicted in Fig. 1 plateau with increasing rarefaction depth, indicating that the sequencing depth and diversity coverage achieved

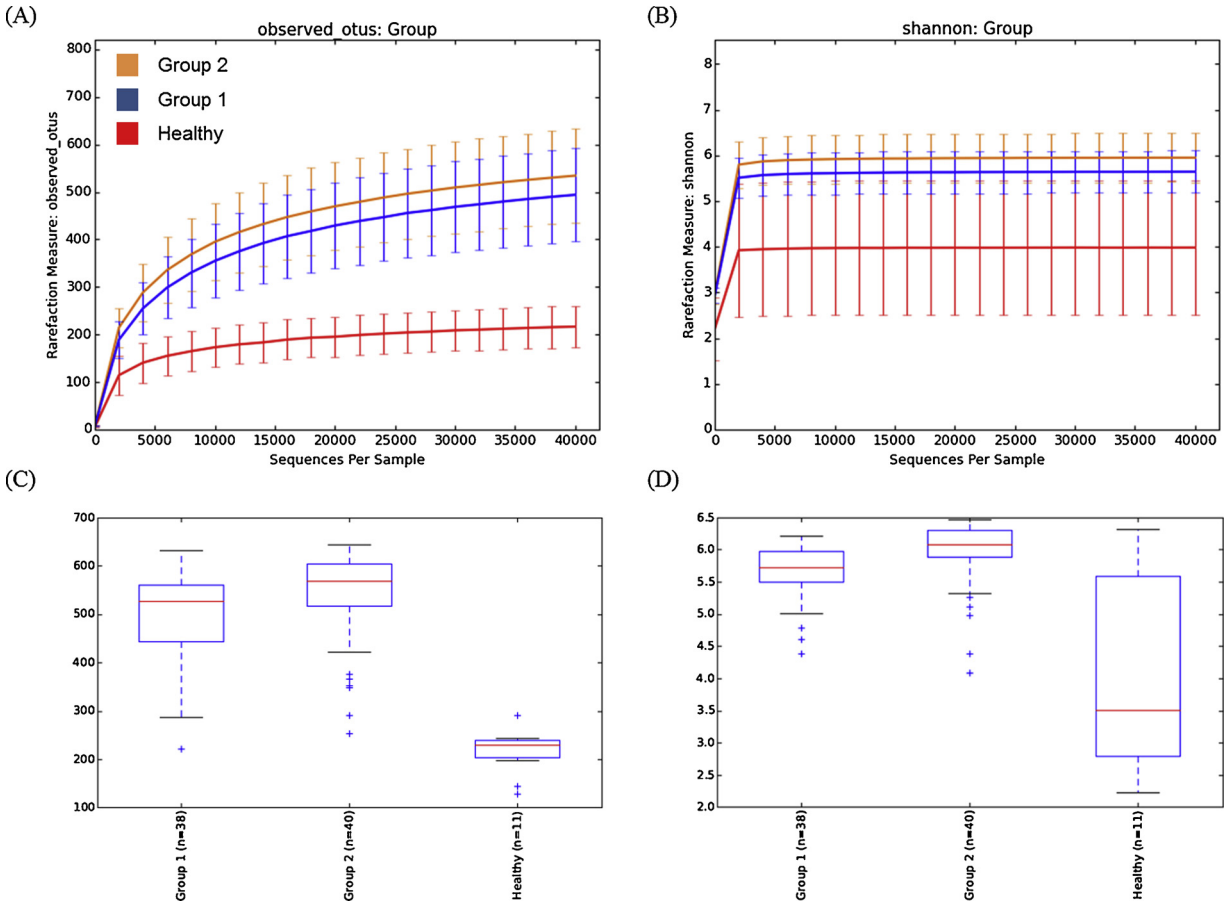


Fig. 1. Alpha diversity by Group, as defined by the number of observed OTUs and Shannon's diversity index: (a) alpha rarefaction curves based on the number of observed OTU's. Each point represents the average number of OTUs identified, and error bars indicate the standard error; (b) (a) alpha rarefaction curves based on Shannon's diversity index. Each point represents the average number of OTUs identified, and error bars indicate the standard error; (c) alpha diversity boxplots based on the number of observed OTU's; (d) alpha diversity boxplots based on Shannon's diversity index.

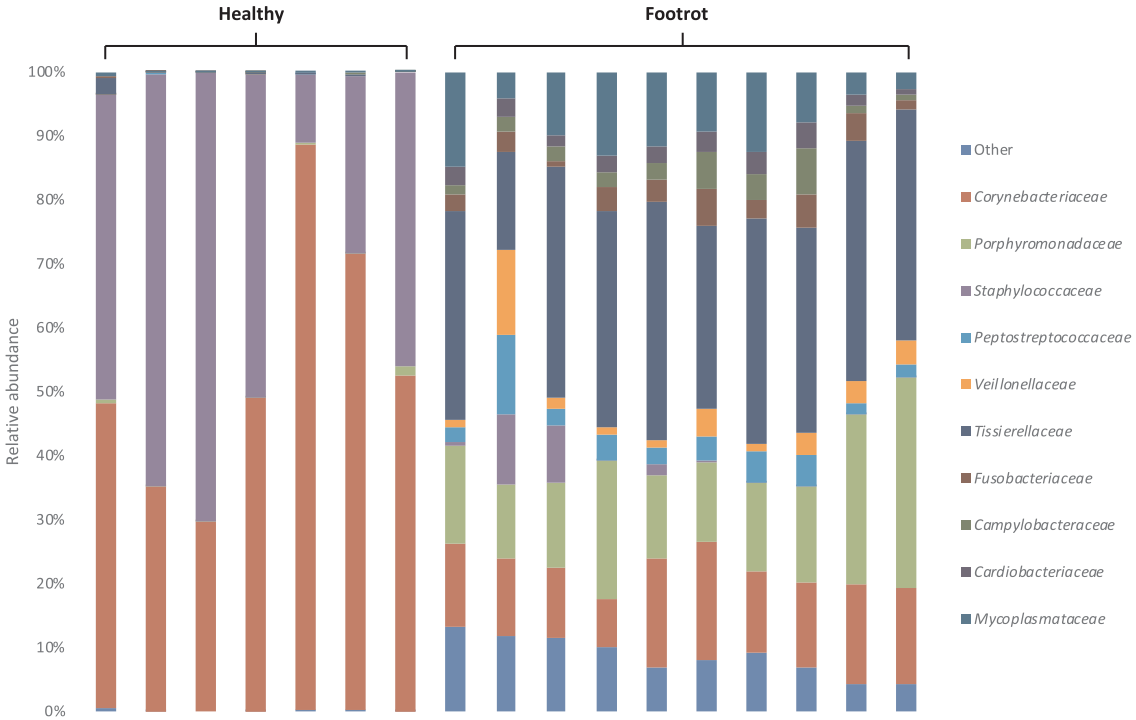
were satisfactory to accurately describe the diversity of the bacterial communities.

Alpha diversity was significantly greater on the feet of footrot-affected sheep than healthy sheep, as defined by the number of OTUs observed ($P = 0.003$) and Shannon's diversity index ($P = 0.003$) (Fig. 1). The mean number of OTUs identified on the feet of sheep in the healthy group was 227 (range 148–308), whilst the mean number of OTUs identified on the feet of sheep in Groups 1 and 2 were 528 (range 255–659) and 566 (range 299–719), respectively. Alpha diversity was not significantly different between the samples collected from sheep in Groups 1 and 2, as defined by the number of observed OTUs ($P = 0.255$). However, Shannon's diversity index was significantly different when comparing alpha diversity between Groups 1 and 2 ($P = 0.036$).

The dominant bacterial families on the feet of healthy sheep were *Actinomycetaceae* (35.9%), *Staphylococcaceae* (20.7%), *Moraxellaceae* (11.0%), *Sphingomonadaceae* (4.3%), and *Microbacteriaceae* (4.3%) (Fig. 2). The dominant bacterial families detected on the feet of footrot-affected sheep in Group 1 were *Tissierellaceae* (33.6%), *Porphyromonadaceae* (15.0%), *Mycoplasmataceae* (10.5%), *Actinomycetaceae* (7.0%), and *Fusobacteriaceae* (4.5%) (Fig. 2). The dominant bacterial families detected on the feet of footrot-affected sheep in Group 2 were *Tissierellaceae* (33.2%), *Porphyromonadaceae* (15.1%), *Actinomycetaceae* (13.9%), *Peptostreptococcaceae* (4.4%) and *Mycoplasmataceae* (3.7%) (Fig. 2).

Beta diversity was assessed after rarefaction using weighted and unweighted UniFrac distances. The PCoA plots generated from both the unweighted and weighted UniFrac matrics indicated a clear separation of the bacterial communities present on the feet of sheep in the healthy group and those present on the feet of sheep in Groups 1 and 2 (Fig. 3). The PCoA plots generated from the unweighted UniFrac distance matrix (Fig. 3a), which considers the presence or absence of taxa only, demonstrated a slight separation of the bacterial communities present on the feet of sheep in Groups 1 and 2. However, there was no apparent separation of the bacterial communities present on the feet of sheep in Groups 1 and 2 in the PCoA plots generated from the weighted UniFrac matrix, which consider the presence or absence of taxa, as well as their relative abundance (Fig. 3b). Non-parametric statistical tests were used to evaluate the UniFrac distance matrices comparing sheep in the healthy group with sheep in Groups 1 and 2. There was a significant difference in the diversity of bacterial communities on the feet of the healthy and footrot-affected sheep for both the weighted (AnoSim, $R = 0.94$, $P < 0.001$; ADONIS, $R^2 = 0.34$, $P < 0.001$) and unweighted (AnoSim, $R = 0.97$, $P < 0.001$; ADONIS, $R^2 = 0.32$, $P < 0.001$) UniFrac distance matrices.

The compositions of the bacterial communities on the feet of footrot-affected sheep were compared between scores. Samples collected from Score 4 lesions (Samples 1 and 13, from Group 1 and Group 2, respectively) were excluded from these analyses as only two samples were collected from such lesions. Alpha diversity did not differ significantly between lesion scores of 1 to 3, as defined by the number of observed OTUs and Shannon's diversity index ($P > 0.05$), nor did beta diversity, according to the unweighted (AnoSim, $R^2 = 0.019$, $P = 0.609$; ADONIS, $R^2 = 0.022$, $P = 0.086$) and weighted (AnoSim, $R^2 = 0.003$, $P = 0.447$; ADONIS, $R^2 = 0.034$, $P = 0.06$) UniFrac distances (Fig. 4; Supplementary Fig. 1). Alpha diversity was significantly greater in the score 0 lesions of footrot-affected sheep than in those of



Month	M	A	M	J	J	A	S	M	A	M	J	J	A	S	O	N	D
Score 1	2	2	2	2	2	2	2	0	1	2	0	0	1	0	0	0	0
Score 2	0	0	0	0	0	0	0	2	1	2	4	7	4	1	2	0	0
Score 3	0	0	0	0	0	0	0	1	6	1	3	1	3	4	5	6	6
Score 4	0	0	0	0	0	0	0	6	0	3	1	0	0	3	1	1	2

Fig. 2. Relative abundance of the dominant bacterial families on the feet of healthy and footrot-affected sheep based on the number of bacterial 16S rRNA sequences. Bacterial families that represent at least one percent of the total data set are depicted. The number of feet represented by each bar, and their respective scores, is depicted below.

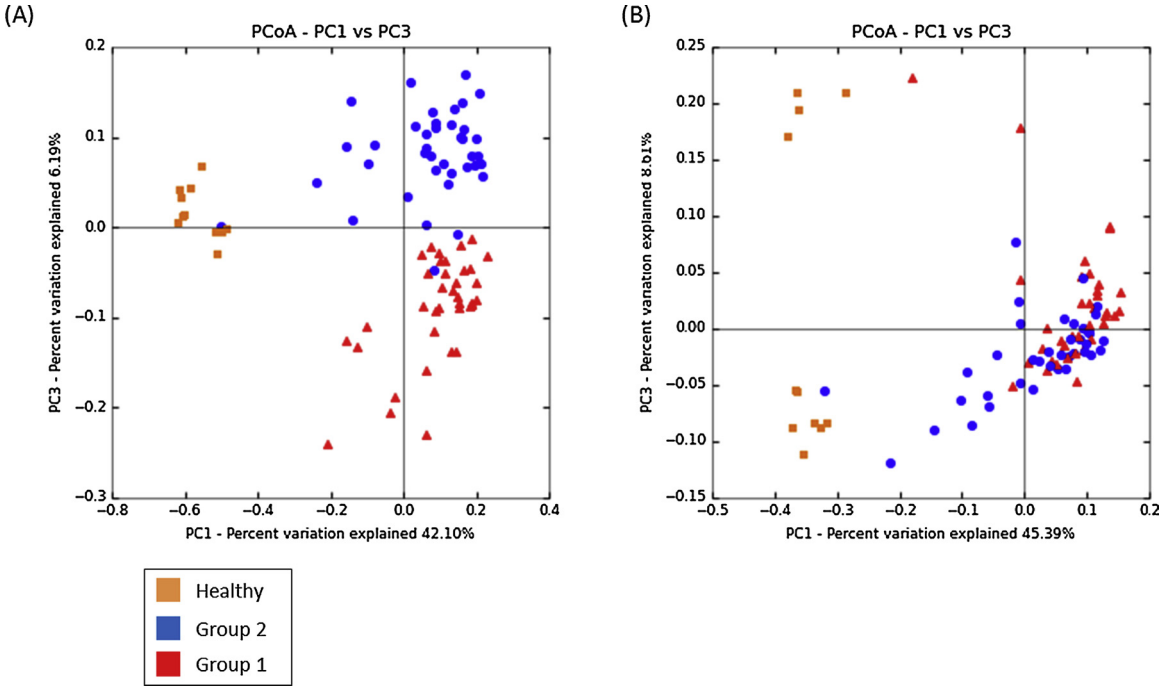


Fig. 3. PCoA plots comparing bacterial communities in each group, based on (a) unweighted and (b) weighted UniFrac distance matrices.

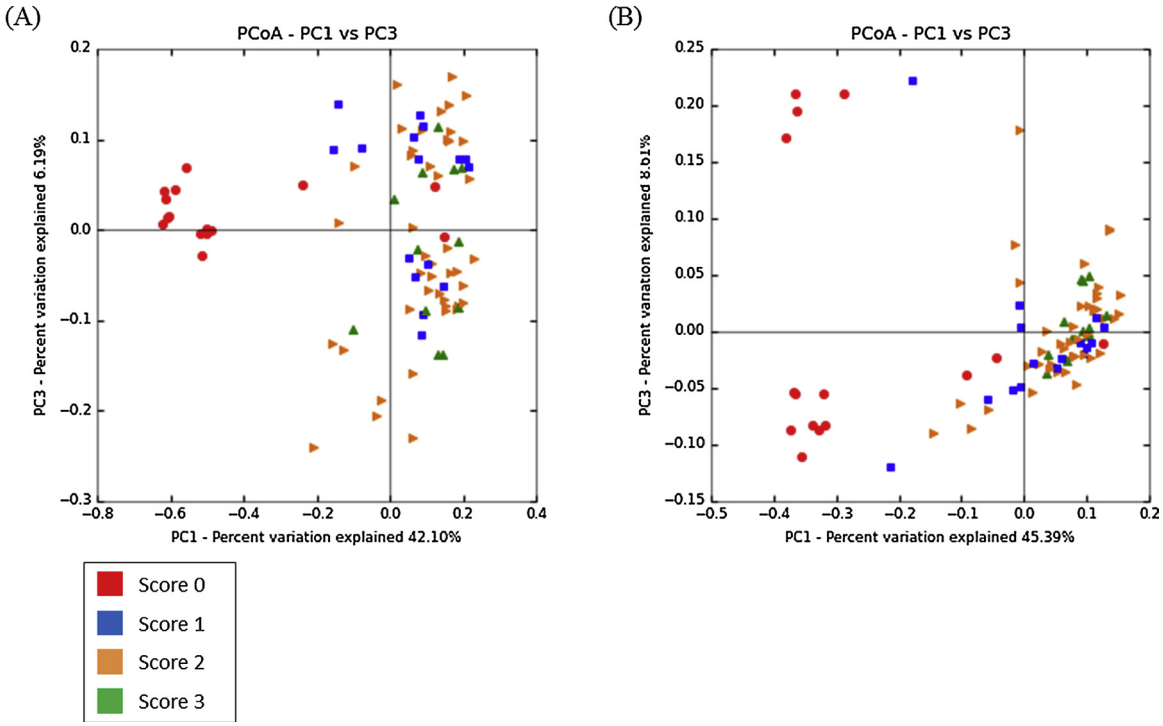


Fig. 4. PCoA plots comparing bacterial communities from different lesion scores in the footrot-affected group, based on (a) unweighted and (b) weighted UniFrac distance matrices.

the healthy sheep, as defined by the number of observed OTUs (P = 0.003). Shannon's diversity index was not significant (P = 0.13). Beta diversity differed significantly between the two groups according to both the unweighted (AnoSim, R^2 = 0.35, P = 0.002; ADONIS, R^2 = 0.71, P = 0.004) and weighted (AnoSim, R^2 = 0.56, P = 0.008; ADONIS, R^2 = 0.26, P = 0.006) UniFrac distance matrices.

Beta diversity was compared between feet with active lesion lesions assigned the same score (scores 1–3). Beta diversity did not differ significantly between feet assigned a lesion score of 1 according to the unweighted (AnoSim, R = 0.10, P = 0.267; ADONIS, R^2 = 0.45, P = 0.183) or weighted (AnoSim, R = 0.05, P = 0.357; ADONIS, R^2 = 0.37, P = 0.605) UniFrac distance matrices. Beta diversity differed significantly between feet assigned a lesion score of 2 according to the both unweighted (AnoSim, R = 0.29, P = 0.001; ADONIS R^2 = 0.31, P = 0.001) and weighted (AnoSim, R = 0.24, P = 0.001; ADONIS, R^2 = 0.33, P = 0.001) UniFrac distance matrices. Beta diversity differed significantly between feet assigned a score of 3 according to the unweighted UniFrac distance matrix (AnoSim, R = 0.68, P = 0.003; ADONIS, R^2 = 0.71, P = 0.003), but not the weighted UniFrac distance matrix (AnoSim, R = 0.37, P = 0.064; ADONIS, R^2 = 0.70, P = 0.075).

To examine variation in the bacterial communities on the feet of each group across the 10-month trial period, alpha diversity was assessed according to time point (month), as defined by the number of observed OTUs and Shannon's diversity index. There was no significant difference ($P > 0.05$) between time points within any of the three groups according either alpha diversity metric.

No taxonomic groups were differentially abundant in samples collected from the feet of sheep in Groups 1 and 2. Consequently, sheep in the healthy group were compared to footrot-affected sheep. Fifteen genera were significantly more abundant on the feet of footrot-affected sheep than on the feet of sheep in the healthy group, including *Porphyromonas* (Gram-negative, anaerobic), *Peptoniphilus* (Gram-positive, anaerobic), *Mycoplasma*, *Anaerococcus* (Gram-positive, anaerobic), *Fusobacterium* (Gram-negative, anaerobic), *Campylobacter* (Gram-negative, microaerophilic), and *Dichelobacter* (Gram-negative, anaerobic) (Fig. 5, Table 2). Six genera were significantly more abundant on the feet of sheep in the healthy group than on the feet of footrot-affected sheep, including *Staphylococcus* (Gram-positive, facultative anaerobic), *Sphingomonas* (Gram-negative, aerobic), *Macrococcus* (Gram-positive, facultative anaerobic), *Pseudoclavibacter* (Gram-positive, aerobic), *Acinetobacter* (Gram-negative, aerobic), and *Wautersiella* (Gram-negative, aerobic) (Fig. 5, Table 2).

4. Discussion

The primary aim of this study was to characterise the bacterial communities on the feet of healthy and footrot-affected Merino sheep and to examine whether the composition of the bacterial community changes over time as a result of the disease process. The bacterial communities on the feet of the footrot-affected sheep (Groups 1 and 2) were taxonomically more diverse than those on the feet of the healthy sheep (Fig. 1). Furthermore, the disease process appeared to trigger a shift in the composition of the bacterial community from predominantly Gram-positive, aerobic bacterial taxa on healthy feet to predominantly Gram-negative, anaerobic taxa on footrot-affected feet (Fig. 2). A similar shift has been reported in other mixed bacterial diseases of both humans and animals, along with an increase in taxonomic diversity, and both appear to coincide with a transition from a healthy state to a state of disease, irrespective of body site (Darveau et al., 2012; Kennedy et al., 2016; Krull et al., 2014; Maboni et al., 2017). The dominant genera in diseased tissue differ according to the disease, species and body site associated with a moderate to severe inflammatory response (Darveau et al., 2012; Kennedy et al., 2016; Krull et al., 2014; Maboni et al., 2017; Stewart, 1989). The composition of the bacterial communities on footrot-affected feet in this study closely resemble those on the feet of cattle with bovine digital dermatitis (BDD), with an increase in the abundance of the families *Mycoplasmataceae*, *Porphyromonadaceae*, and *Tissierellaceae* (Krull et al., 2014; Nielsen et al., 2016). The inflammatory response is responsible for much of the tissue damage observed in polymicrobial diseases like footrot. Studies on this disease have focussed on the role of *Fusobacterium necrophorum* which colonises the epidermis following

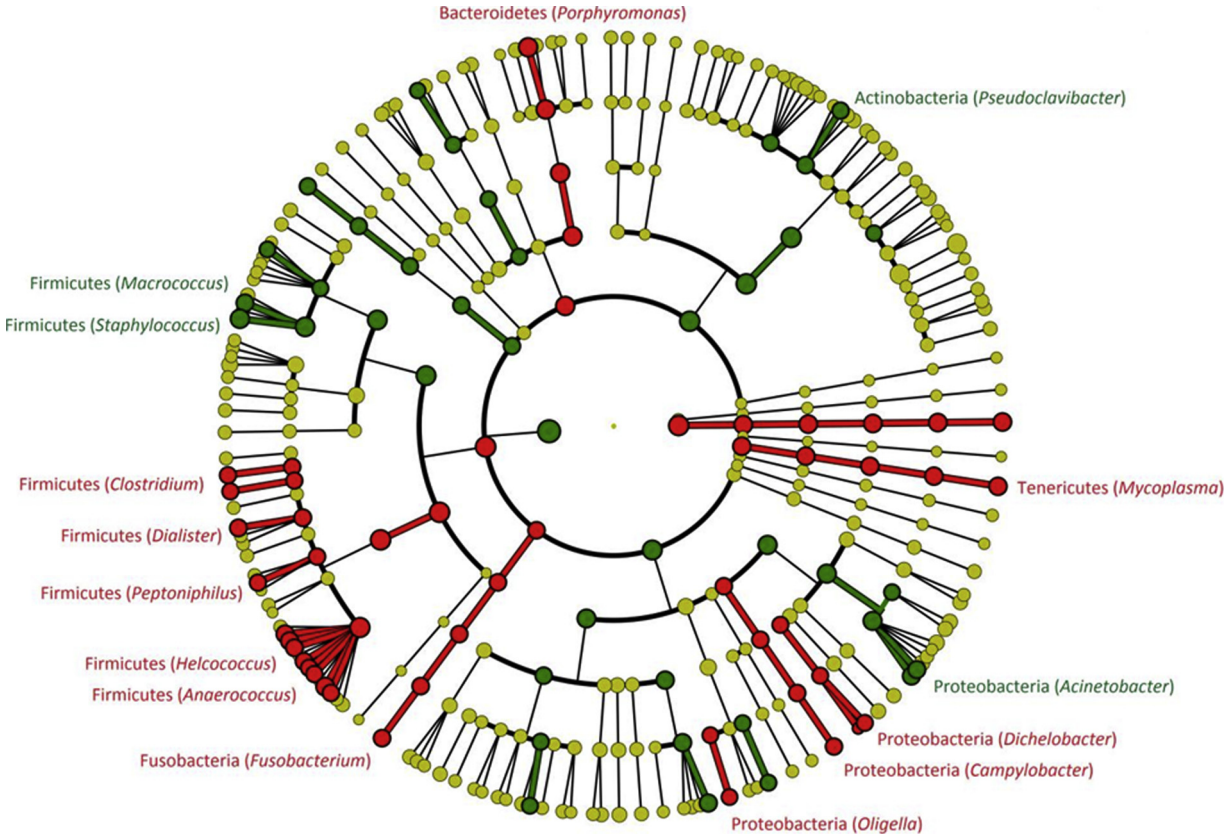


Fig. 5. Cladogram of the bacterial taxa that had a significant Kruskal-Wallis test result ($P < 0.05$) and an LDA score ≥ 2.0 as determined by LEfSe. Bacterial phyla are represented by the central ring of the cladogram, and each subsequent ring represents the next taxonomic level. Taxa that were significantly more abundant on the feet of sheep in the healthy group or footrot-affected group are represented in green and red, respectively, and non-significant taxa are represented in yellow. The labels indicate significant phyla, with genera listed in parentheses (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

environmental predisposition, after which it expresses a leukocytic exotoxin that inhibits or disrupts the immune response, enabling both itself and other taxa to proliferate (Roberts and Egerton, 1969; Stewart, 1989). In the present study, *Fusobacterium necrophorum* was found to be preferentially abundant on the feet of footrot-affected sheep. However, we identified other taxa on the feet of the footrot-affected sheep that may also be of significance to the disease process.

Fifteen bacterial genera were preferentially abundant on the feet of footrot-affected sheep according to the LEfSe analysis (Table 2, Fig. 5), including *Porphyromonas*. This genus has previously been implicated in the pathogenesis of ovine footrot or reported to be abundant in necrotic material collected from foot lesions (Jimenez et al., 2003; Maboni et al.,

Table 2
Biomarker genera identified using LEfSe. All taxa were significantly more abundant in the class of interest (Kruskal-Wallis $P < 0.05$) and had an LDA score > 3. Phylum, Class, Order, and Family are also listed for each biomarker genus.

Group	Phylum	Class	Order	Family	Genus	LDA Score
Healthy	Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus*	4.19
Healthy	Proteobacteria	Alphaproteobacteria	Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*	3.66
Healthy	Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Macrococcus*	3.63
Healthy	Actinobacteria	Actinobacteria	Actinomycetales	*Microbacteriaceae*	*Pseudoclavibacter*	3.37
Healthy	Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*	*Acinetobacter*	3.37
Healthy	Bacteroidetes	Flavobacteriia	Flavobacteriales	*Weeksellaceae*	*Wautersiella*	3.10
Footrot	Bacteroidetes	Bacteroidia	Bacteroidales	*Porphyromonadaceae*	*Porphyromonas*	4.17
Footrot	Tenericutes	Mollicutes	Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*	3.83
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*1_68*	3.69
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*Anaerococcus*	3.50
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*GW_34*	3.50
Footrot	Fusobacteria	Fusobacteriia	Fusobacteriales	*Fusobacteriaceae*	*Fusobacterium*	3.48
Footrot	Proteobacteria	Epsilonproteobacteria	Campylobacterales	*Campylobacteraceae*	*Campylobacter*	3.42
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*Tepidimicrobium*	3.36
Footrot	Firmicutes	Clostridia	Clostridiales	*Veillonellaceae*	*Dialister*	3.32
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*Gallicola*	3.28
Footrot	Firmicutes	Clostridia	Clostridiales	*Tissierellaceae*	*Helcococcus*	3.27
Footrot	Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	3.26
Footrot	Proteobacteria	Betaproteobacteria	Burkholderiales	*Alcaligenaceae*	*Oligella*	3.17
Footrot	Proteobacteria	Gammaproteobacteria	Cardiobacteriales	*Cardiobacteriaceae*	*Dichelobacter*	3.00

2017; Nattermann et al., 1993; Piriz Duran et al., 1990). The extent to which *Porphyromonas* contributes to the expression of ovine footrot is unknown but it may have a significant role. An abundance of *Porphyromonas* in footrot lesions was recently found to be significantly associated with high expression levels of pro-inflammatory cytokines (Maboni et al., 2017), while *Porphyromonas levii* (formerly *Bacteroides levii*) was shown to enhance the severity of BDD lesions (Walter and Morck, 2002). Recent metagenomic surveys of BDD lesions have found *Porphyromonas* to be abundant in BDD lesions (Krull et al., 2014; Nielsen et al., 2016). *P. gingivalis*, a member of the normal human oral bacterial community, is regarded as a 'keystone' species in the pathogenesis of periodontal disease which is also associated with a mixed bacterial infection (Darveau et al., 2012). The bacterium is able to manipulate the hosts' immune response and inhibit leukocyte-mediate killing mechanisms, leading to unrestrained proliferation of other bacterial taxa (Wang et al., 2010). Further investigation is required to determine the extent to which *Porphyromonas* might help to trigger a state of dybiosis on the feet of footrot-affected sheep.

The composition of the bacterial communities on footrot-affected feet did not differ significantly between active lesions of different scores (1 to 3) according to any of the alpha or beta diversity metrics (Fig. 4; Supplementary Fig. 1). This finding differs to that of previous studies, in which distinct bacterial communities were observed on feet with mild lesions (scores 1 or 2) and those on feet with underrun lesions (scores 3 or 4) (Calvo-Bado et al., 2011; Maboni et al., 2017). Distinct bacterial communities have also been reported in skin biopsies collected from the mild and severe BDD lesions (Krull et al., 2014; Nielsen et al., 2016). Differences in sampling strategies between studies may explain this. We collected swabs from the surface of the active margin of each lesion or the interdigital skin if there was no lesion, with the aim of identifying taxa that are most likely to be driving the progression of foot lesions. In previous studies (Calvo-Bado et al., 2011; Maboni et al., 2017), the samples consisted of interdigital skin biopsies collected post mortem, and the data obtained possibly reflect the skin microbiome, including bacteria present in the deeper layers of the epidermis, rather than that of the active underrunning lesion of virulent footrot. Furthermore, previous studies have analysed samples collected from the feet of different sheep with different lesions scores; thus, the reported variation between lesion scores may reflect differences between flocks and geographic region. In the present study, a longitudinal design was used, and samples were collected from different lesion scores on the same feet across time. Samples were collected from four score 0 lesions in the footrot-affected group during the course of the trial, which were observed during the autumn and winter months (Table 1). Surprisingly, a state of dysbiosis was also apparent in these inactive lesions, as the composition of the bacterial communities in these lesions did not differ significantly to those present in active lesions ($\geq$ score 1). One score 0 lesion from the footrot-affected group clustered with those of the healthy group in the PCoA plot (Fig. 4), which indicates that on this foot at this time point the bacterial community resembled that of a healthy foot. Nevertheless, the persistence of this state of dysbiosis may be a predisposing factor, as these lesions all recrudesced with the advent of favourable environmental conditions in spring (Table 1).

Beta diversity was compared between feet assigned the same lesion score. Beta diversity did not differ significantly between feet assigned a score of 1, but did differ significantly between feet assigned a lesion score of 2, according to both the unweighted and weighted UniFrac distance matrices. Beta diversity differed significantly between feet assigned a lesion score of 3 according to the unweighted UniFrac distance matrix only, which indicated that the bacterial communities differed by the number of taxa identified, but that the overall composition of the community was similar when relative abundance was taken into consideration. Variation between feet is to be expected given that the lesion scoring system used in this study describes broad changes in the appearance of foot lesions and does not capture minor variations between different feet. Furthermore, such variation could also be attributed to factors other than natural biological variability, including the amount of lesion material collected from each foot, PCR bias, and the quality and quantity of sequencing data obtained from each sample.

The fact that the composition of the bacterial community at the active margin was similar in both mild and severe lesions suggests that the same taxa might be driving the progression of foot lesions at both the early and latter stages of disease, which means that the virulence attributes of the particular *D. nodosus* strains present would at least partially determine the extent of the underrunning. However, given the sampling strategy employed in this study, our results largely reflect the bacterial taxa present in the superficial layers of the lesion, some of which might not contribute to the disease process. There may also be differences in the bacterial taxa present in the deeper layers of the lesion. Future studies would benefit from the examination histological sections collected from the same lesions sampled for metagenomic analyses.

Amplicon-based metagenomics includes potential biases, from sample collection and DNA extraction, through to PCR amplification and bioinformatics strategy (Chakravorty et al., 2007; Walker et al., 2015), which must be considered when interpreting the data. We chose to amplify the V3-V4 region of the bacterial 16S rRNA gene, as it can discriminate between clinically important genera (Chakravorty et al., 2007) and to enable comparisons with previous metagenomic studies of footrot and other polymicrobial diseases of the ruminant foot (Krull et al., 2014; Maboni et al., 2017; Nielsen et al., 2016). Previous studies have also reported the detection of *D. nodosus* when targeting the V3-V4 region (Maboni et al., 2017; Nielsen et al., 2016). The V1-V3 region was deemed unsuitable as *D. nodosus* was not detected when targeting the V1-V3 region with the primers 27 F and 1525R, due to the presence of two primer mismatches in the binding region of the 27 F primer (Calvo-Bado et al., 2011). In the present study, we were also able to detect *D. nodosus* by targeting the V3-V4 region using the primers 341 F and 806R. Consequently, we recommend this region of the bacterial 16S rRNA gene as a suitable target for future metagenomic studies of ovine footrot.

We acclimated sheep for one month on pasture prior to the study because bacterial communities may vary according to geographic location (Calvo-Bado et al., 2011). Two groups of sheep with footrot were included to obtain some diversity in the types of *D. nodosus* that may be present, this taxon being an obligate parasite and not originating from the environment. We found no significant differences between the two groups, which suggests that the influence of background geography was diminished following acclimation. Nevertheless, the aetiology of footrot may indeed differ to some extent between flocks and geographic regions, thus the extent to which the results of the present study can be applied to the Merino breed more generally may be limited. Investigation of the aetiology of footrot in Merino flocks in different geographic locations was beyond the scope of this study, which was intended to provide baseline data only. However, future studies would benefit from the inclusion of flocks in different geographic regions, which would help to inform the development of novel treatment and control measures.

The expression of footrot is influenced by climatic factors, namely temperature and moisture (Graham and Egerton, 1968). In Australia, lesions tend to be most severe during the spring and autumn spread periods and less so in the dry summer and cold winter. We examined the longitudinal variation of the bacterial communities on the feet of both footrot-affected groups of sheep from March through to December, capturing spread-periods (spring, autumn) and non-spread periods (winter). Foot lesions were somewhat less severe during the winter months (June – August). However, the composition of the bacterial communities did not differ significantly over time according to any of the alpha- or beta-diversity metrics used in this study. This suggests that temporal variations in lesion severity were due to factors other than changes in the composition of the bacterial community.

Further investigation is required to establish which of the fifteen genera found to be preferentially abundant in footrot lesions contribute to the disease process, and the means by which they contribute. It is possible that some of these genera play no role in the disease process, and that the lesion simply provides an ideal environment for them to proliferate. However, those found to trigger a shift in the composition of the bacterial community, alone or in combination with other taxa, would be a logical target for new footrot treatment and control measures, such as vaccines. Vaccines targeting *F. necrophorum* have been evaluated for the prevention and treatment of ovine footrot (Berg, 1990; Thompson et al., 1995), and have been shown to have both therapeutic and protective effects, with one study reporting a curative rate of 87% of affected feet and a preventive effect of 69% (Thompson et al., 1995). The use of vaccines targeting *Porphyromonas* for the control and treatment of footrot has not yet been investigated; however, vaccines targeting this genus have been investigated for the prevention of periodontal disease in both animals and humans, with promising results (Hardham et al., 2005; O'Brien-Simpson et al., 2016). In contrast to footrot vaccines targeting *D. nodosus*, which require isolation and serotyping of the infecting *D. nodosus* strain(s) in order to formulate approriate serogroup-specific vaccine(s) (Dhungyel et al., 2013), vaccines targeting opportunistic pathogens like *Porphyromonas* might provide effective control of footrot without the need for costly and laborious diagnostic tests, if it can be shown that the same genera are abundant in all flocks with footrot, irregardless of breed and geographic location.

5. Conclusion

Different bacterial communities were identified on the feet of healthy and footrot-affected Merino sheep. Gram-positive, aerobic taxa dominated on the feet of healthy sheep, compared to predominantly Gram-negative, anaerobic species on the feet of footrot-affected sheep. In the latter, the composition of the bacterial community at the active margins of the lesions did not differ significantly between lesion scores or over time, which suggests that similar taxa drive both the early and later stages of the disease. The roles of particular taxa should be investigated in future studies together with investigation of similarities and differences between bacterial communities at the active margins of lesions and the broader interdigital skin.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.08.001.

References

Amies, C.R., 1967. A modified formula for the preparation of stuart's transport medium. Can. J. Public Health 58, 296–300.
Andrews, S., 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data.
Berg, J.N., 1990. The use of vaccines against *fusobacterium necrophorum* for control of chronic foot rot in sheep. Symposium on Diseases of Small Ruminants. Oregon State University, Corvallis, OR 115.
Beveridge, W.I.B., 1941. Foot-rot in sheep: a transmissible disease due to infection with *Fusiformis nodosus* (. sp.). Studies on its cause, epidemiology, and control. Australian Council for Scientific and Industrial Research Bulletin 140, 1–56.
Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57.
Borsanelli, A.C., Lappinb, D.F., Viora, L., Bennett, D., Dutra, I.S., Brandt, B.W., Riggio, M.P., 2018. Microbiomes associated with bovine periodontitis and oral health. Vet. Microbiol. 218, 1–6.
Buller, N., Eamens, G., 2014. Ovine footrot. Australian and New Zealand Standard Diagnostic Procedure (Sub-Committee on Animal Health Laboratory Standards (SCAHLS)).
Calvo-Bado, L.A., Oakley, B.B., Dowd, S.E., Green, L.E., Medley, G.F., Ul-Hassan, A., Bateman, V., Gaze, W., Witcomb, L., Grogono-Thomas, R., Kaler, J., Russell, C.L., Wellington, E.M.H., 2011. Ovine pedomics: the first study of the ovine foot 16S rRNA-based microbiome. ISME J. 5, 1426–1437.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266–267.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Tumbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U.S.A. 108, 4516–4522.
Chakravorty, S., Helb, D., Burday, M., Connell, N., Alland, D., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods 69, 330–339.
Darveau, R.P., Hajishengallis, G., Curtis, M.A., 2012. *Porphyromonas gingivalis* as a potential community activist for disease. J. Dent. Res. 91, 816–820.
Dhungyel, O., Schiller, N., Eppleston, J., Lehmann, D., Nilon, P., Ewers, A., Whittington, R., 2013. Outbreak-specific monovalent/bivalent vaccination to control and eradicate virulent ovine footrot. Vaccine 31, 1701–1706.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics (Oxford) 26, 2460–2461.
Egerton, J.R., Roberts, D.S., 1971. Vaccination against ovine foot-rot. J. Comp. Pathol. 81, 179–185.
Egerton, J.R., Roberts, D.S., Parsonson, I.M., 1969. The aetiology and pathogenesis of ovine foot-rot: I. A histological study of the bacterial invasion. J. Comp. Pathol. 79, 207–215.
Emery, D.L., Stewart, D.J., Clark, B.L., 1984. The comparative susceptibility of five breeds of sheep to foot-rot. Aust. Vet. J. 61, 85–88.
Graham, N.P.H., Egerton, J.R., 1968. Pathogenesis of ovine foot-rot: the role of some environmental factors. Aust. J. Sci. 44, 235–240.
Hardham, J., Reed, M., Wong, J., King, K., Laurinat, B., Sfintescu, C., Evans, R.T., 2005. Evaluation of a monovalent companion animal periodontal disease vaccine in an experimental mouse periodontitis model. Vaccine 23, 3148–3156.
Jimenez, R., Piriz, S., Martin-Palomino, P., Mateos, E., Vadillo, S., 2003. Aetiology of ovine footrot in the portuguese region of alto alentejo. J. Vet. Med. Ser. B 50, 118–120.
Kennedy, R., Lappin, D.F., Dixon, P.M., Buijs, M.J., Zura, E., Crielaard, W., O'Donnell, L., Bennett, D., Brandt, B.W., Riggio, M.P., 2016. The microbiome associated with equine peridontitis and oral health. Vet. Res. 47.
Krull, A.C., Shearer, J.K., Gorden, P.J., Cooper, V.L., Phillips, G.J., Plummer, P.J., 2014. Deep sequencing analysis reveals temporal microbiota changes associated with development of bovine digital dermatitis. Infect. Immun. 82, 3359–3373.
Lane, J., Jubb, T., Shephard, R., Webb-Ware, J., Fordyce, G., 2015. Priority List of Endemic Diseases for the Red Meat Industries (Meat and Livestock Australia).
Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169–172.
Maboni, G., Blanchard, A., Frosth, S., Stewart, C., Emes, R., Tötemeyer, S., 2017. A distinct bacterial dysbiosis associated skin inflammation in ovine footrot. Sci. Rep. 7.
Martin, J.M., Zenilman, J.M., Lazarus, G.S., 2010. Molecular microbiology: new dimensions for cutaneous biology and wound healing. J. Invest. Dermatol. 130, 38–48.
McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618.
Muyzer, G., De Waal, E.D., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
Nattermann, H., Barwisch, R., Briedermann, L., 1993. Experimental reproduction of footrot in Merino mutton sheep, using *Bacteroides nodosus* isolates from Moufflon

(*Ovis ammon musimon*). Monatshefte für Veterinärmedizin 48, 651–655.
Nielsen, M.W., Strube, M.L., Isbrand, A., Al-Medrasi, W.D.H.M., Boye, M., Jensen, T.K., Klitgaard, K., 2016. Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. Vet. Microbiol. 186, 139–149.
O'Brien-Simpson, N.M., Holden, J.A., Lenzo, J.C., Tan, Y., Brammar, G.C., Walsh, K.A., Singleton, W., Orth, R.K.H., Slakeski, N., Cross, K.J., Darby, I.B., Becher, D., Rowe, T., Morelli, A.B., Hammet, A., Nash, A., Brown, A., Ma, B., Vingadassalom, D., McCluskey, J., Kleanthous, H., Reynolds, E.C., 2016. A therapeutic Porphyromonas gingivalis gingipain vaccine induces neutralising IgG1 antibodies that protect against experimental periodontitis. Npj. Vaccines 1 16022.
Piriz Duran, S., Cuenca Valera, R., Valle Manzano, J., Vadillo Machota, S., 1990. Isolation and identification of anaerobic bacteria from ovine foot rot in Spain. Res. Vet. Sci. 49, 245–247.
Roberts, D.S., Egerton, J.R., 1969. The aetiology and pathogenesis of ovine footrot: II. The pathogenic association of *Fusiformis nodosus* and *Fusiformis necrophorus*. J. Comp. Pathol. 79, 217–227.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.
Stehlikova, Z., Kostovcik, M., Kostovcikova, K., Kverka, M., Juzlova, K., Rob, F., Hercogova, J., Bohac, P., Pinto, Y., Uzan, A., Koren, O., Tlaskalova-Hogenova, H., Zakostelska, Z.J., 2019. Dysbiosis of skin microbiota in psoriatic patients: co-occurrence of fungal and bacterial communities. Front. Microbiol. 10, 438.
Stewart, D.J., 1979. The role of elastase in the differentiation of *Bacteroides nodosus* infections in sheep and cattle. Res. Vet. Sci. 27, 99–105.
Stewart, D.J., 1989. Footrot of sheep. In: Egerton, J.R., Yong, W.K., Riffkin, G.G. (Eds.), Eds.) Footrot and Foot Abscess of Ruminants. CRC Press, Boca Raton, pp. 5–45.
Stewart, D.J., Peterson, J.E., Vaughan, J.A., Clark, B.L., Emery, D.L., Caldwell, J.B., Kortt, A.A., 1986. The pathogenicity and cultural characteristics of virulent, intermediate and benign strains of *Bacteroides nodosus* causing ovine foot-rot. Aust. Vet. J. 63, 317–326.
Thompson, J.M., Hansen, D.E., McCoy, R.D., 1995. Efficacy of a vaccine against *Fusobacterium necrophorum* for control of foot rot in sheep. Sheep & Goat Research Journal 11, 51–53.
Walker, A.W., Martin, J.C., Scott, P., Parkhill, J., Flint, H.J., Scott, K.P., 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 3.
Walter, M.R.V., Morck, D.W., 2002. Chemotaxis, phagocytosis, and oxidative metabolism in bovine macrophages exposed to a novel interdigital phlegmon (foot rot) lesion isolate, Porphyromonas levii. Am. J. Vet. Res. 63, 757–762.
Wang, M., Krauss, J.L., Domon, H., Hosur, K.B., Liang, S., Magotti, P., Triantafilou, M., Triantafilou, K., Lambris, J.D., Hajishengallis, G., 2010. Microbial hijacking of complement-toll-Like receptor crosstalk. Sci. Signal. 3, 9.